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UTILITY PATENT APPLICATION TRANSMITTAL <small>(Only for new nonprovisional applications under 37 C.F.R. § 1.53(b))</small>		Attorney Docket No.	UCSD0-078-2
		First Inventor or Application Identifier	EDWARD A. DENNIS
		Title	CLONED HUMAN LYSOPHOSPHOLIPASE
		Express Mail Label No.	EJ079900564US

APPLICATION ELEMENTS <small>See MPEP chapter 600 concerning utility patent application contents.</small>		ADDRESS TO: Assistant Commissioner for Patents Box Patent Application Washington, DC 20231	
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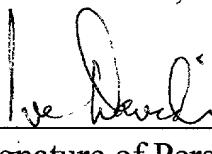
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Edward Dennis
Attorney's Docket No.: UCSD0-078-2
Title: CLONED HUMAN LYSOPHOSPHOLIPASE
UCSD Case No.: SD98-078-2

VERIFIED STATEMENT UNDER 37 CFR §1.9(d) AND §1.27(d)
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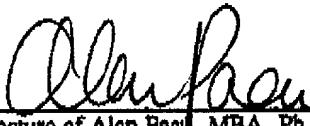
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2004/0203 - P0101

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2. THE REGENTS OF THE UNIVERSITY OF CALIFORNIA is a University of higher education of the State of California and a nonprofit organization.
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5. I acknowledge the duty under 37 CFR §1.28(b) to file, in this application or any patent issuing thereon, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of any issuance fee or any maintenance fee due after that date on which status as a small entity is no longer appropriate.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under §1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent(s) issuing thereon.

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Date


Signature of Alan Paau, MBA, Ph.D.
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**APPLICATION FOR
UNITED STATES PATENT**

IN THE NAME OF

EDWARD A. DENNIS

AND

AIJUN WANG

FOR

CLONED HUMAN LYSOPHOSPHOLIPASE

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Date of Deposit: 12/09/99

CLONED HUMAN LYSOPHOSPHOLIPASE

5

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to enzymes that hydrolyze lysophospholipids in tissues.

The present invention particularly concerns a new, cloned human brain

10 lysophospholipase, its kinetic parameters and of its potential role in treatment of disease states having elevated concentrations of lysophospholipids, including atherosclerosis, hyperlipidemia, lethal dysrhythmias in myocardial ischemia and segmental demyelination of peripheral nerves.

15 Description of Related Art

Lysophospholipases (LysoPLA) are important enzymes that hydrolyze lysophospholipids (LysoPL). LysoPLs are detergent-like intermediates in phospholipid metabolism and play essential roles in many physiological and pathological processes.

20 Lysophosphatidylcholine (LysoPC), a normal constituent of cell membranes, may act as a lipid messenger, transducing signals initiated from membrane receptors. Exogenous addition of LysoPC to cultured cells regulates the expression and/or activity of a variety of proteins including nitric oxide synthase, IL-1 β , transcription factor nuclear factor-kappa B, activator protein 1, c-Jun N-terminal kinase, heparin-binding epidermal growth factor-like growth factor, cellular adhesion molecule-1 and many other proteins. LysoPC also modulates 25 intracellular Ca 2 / levels, stimulates the release of arachidonic acid in human endothelial cells and rat mesangial cells through a pathway that involves the activation of PKC and cPLA2.

Lysophosphatidic acid (LPA), the simplest natural occurring LysoPL, is a multifunctional phospholipid messenger that evokes a variety of biological responses, ranging from platelet

aggregation to smooth muscle contraction, from cell proliferation and differentiation to focal adhesion assembly and stress fiber formation. Such diversified biological responses to LPA appear to involve the activation of a specific G-protein coupled receptor, which in turn couples to multiple independent effector pathways including the small GTP-binding proteins 5 Ras and Rho. Indeed, LPA-specific receptors were cloned recently and identified as members of the G-protein coupled receptors. Increased LysoPL levels have been detected in a variety of disease states including atherosclerosis, hyperlipidemia, lethal dysrhythmias in myocardial ischemia and segmental demyelination of peripheral nerves. Accumulation of LysoPL can perturb the activities of many membrane-bound signal-transducing enzymes, distort cell 10 membrane integrity and even lead to cell lysis.

These increased LysoPL levels are believed to result from the dysfunction of LysoPL-regulating enzymes. Since LysoPLs play such diversified roles, their levels must be strictly regulated for proper cell function and survival. LysoPLA, which controls LysoPL levels 15 through hydrolysis, has been identified in a variety of cells and tissues. Recently, a rat and a mouse LysoPLA have been sequenced, cloned and expressed. These two enzymes (both of 25 kDa molecular mass) share high sequence homology and exhibit similar properties. They are new members of the K/L hydrolase family with a catalytic site composed of Ser-119, Asp-174 and His-208.

20

SUMMARY OF THE INVENTION

The present invention contemplates a cDNA segment encoding a lysophospholipid-specific human lysophospholipase enzyme protein molecule having 690 deoxyribonucleotide base pairs from position 48 to position 741 of SEQ ID NO:1, as shown in Figure 1, and 25 modifications thereof that encode active human lysophospholipase molecules.

More preferably, the invention contemplates a deoxyribonucleic acid sequence for a

cDNA segment encoding a lysophospholipid-specific human brain lysophospholipase enzyme protein molecule, having a sequence of 690 deoxyribonucleotide base pairs from position 48 to position 741 of SEQ ID NO:1, as shown in Figure 1, and modifications thereof that encode active human lysophospholipase molecules.

5

The present invention also contemplates a recombinant lysophospholipid-specific human brain lysophospholipase enzyme protein molecule, having a single 25kDa polypeptide of 230 amino acid residues in length, and a catalytic activity site triad at Ser-119, Asp-174 and His-208, and active modifications thereof.

10

More preferably, an amino acid residue sequence for a recombinant lysophospholipid - specific human brain lysophospholipase enzyme protein molecule having amino acid residues from amino acid residue position 1 to amino acid residue position 230, of SEQ ID NO: 2, as shown in Figure 1, and active modifications thereof.

15

The present invention further relates to a contemplated method of inhibiting catalytic activity of a recombinant lysophospholipid-specific human brain lysophospholipase enzyme protein molecule by exposing the lysophospholipase enzyme protein molecule to a solution containing methyl arachidonyl fluorophosphates.

20

In the most preferred embodiment, the inhibiting that results from the exposing is irreversible.

25

In another, most preferred embodiment, is contemplated a method for treating subjects with diseases cased by increased levels of lysophospholipids, the method comprising supplying a recombinant lysophospholipid-specific lysophospholipase enzyme to an enzyme-deficient subject. The method of supplying can be administered by infusion. More

preferably, the supplying is by gene augmentation therapy.

Still further embodiments and advantages of the invention will become apparent to those skilled in the art upon reading the entire disclosure contained herein.

5

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. shows the cDNA and translated amino acid sequences of human brain LysoPLA. The cDNA and amino acid residues that are different between the human and mouse enzymes are in bold, the catalytic triad is marked by *. The primers that were used to clone the human enzyme are indicated by the lines above the cDNA sequences, and the sequences refer to the sequences in the experimental procedures. The 5' and 3' untranslated cDNA sequences were obtained from GenBank.

15 **Figure 2.** is a depiction of SDS-PAGE of human brain LysoPLA. Human recombinant LysoPLA (28 kDa) was strongly induced by IPTG in *E. coli* transformed with PET28a+/hLysoPLA (lane 2), as compared to that of *E. coli* before IPTG induction (lane 1). Lane 3 is the purified human recombinant LysoPLA with the His•Tag attached, and lane 4 shows the protein with the His•Tag removed by thrombin digestion as demonstrated by the 20 protein band shift.

25 **Figure 3.** demonstrates the activity of human LysoPLA as a function of LysoPC concentration. The substrate was prepared as a 20 U concentrated suspension, then diluted in the assay buffer to the desired final concentration, as indicated. 140 ng of recombinant human brain LysoPLA were added to the 1 U substrate to start the reaction at 40°C for 2, 5 or 10 min. The specific activity of lysophospholipase for each substrate concentration was obtained from the activity (nmol/mg) vs. time plot. Data were fit to Michaelis-Menten equation (dotted line) and

to Hill model (solid line). Each data point represents an average of three independent experiments, each in triplicate.

Figure 4. is a kinetic analysis of human recombinant LysoPLA. LysoPLA activity was determined as a function of the mol% of LysoPC achieved either by fixing the LysoPC (1.125 mM) and varying the TX-100 concentration (A), or by fixing TX-100 (1 mM) and varying the LysoPC substrate concentration (B), or by fixing the mole fraction (surface concentration) of LysoPC, as indicated by the numbers in C. Each data point represents an average of four determinations in A, and an average of two independent experiments, each in triplicate in B and C. Data in A and B were fitted assuming Michaelis-Menten kinetics, and the inserts are the linear fits of the double reciprocal plots. Lines in C are the global fits using Equation 1.

Figure 5. demonstrates inhibition of human recombinant LysoPLA by MAFP. LysoPLA activity was assayed in the presence of different concentrations of MAFP, and expressed as percentage of the activity in the absence of the inhibitor. The data were fit to a single exponential decay.

Figure 6A. refers to the tissue distribution of human LysoPLA in the heart, brain, placenta, lung, liver skeletal muscle, kidney and pancreas (lanes 1 - 8 respectively).

Figure 6B. refers to the tissue distribution of human LysoPLA mRNA for more specific brain regions, glands and tissues shown in Figure 6B.

Figure 6C. refers to the textual description of the tissue distribution of human LysoPLA as exemplified in Figure 6B.

Figure 6D. is a Western blot showing the protein levels of LysoPLA in 70 μ g of protein from

human brain, heart, liver, lung kidney, testis, ovary and placenta (lanes 1 -8 respectively) 5ng of purified recombinant human LysoPLA is in lane 9 as a control.

DETAILED DESCRIPTION OF THE INVENTION

5 Abbreviations

	BEL	bromoeno1 lactone;
	BSA	bovine serum albumin;
	cPLA2	cytosolic Ca 2 /-dependent phospholipase A2;
	DPPC	1-palmitoyl-2-palmitoyl-sn-glycero-3-phosphorylcholine;
10	hLysoPLA	human lysophospholipase;
	iPLA2	Ca 2 /-independent phospholipase A2
	IPTG	isopropyl thio-L-D-galactoside;
	LysoPC	1-palmitoyl-sn-glycero-3-phosphorylcholine;
	LysoPL	lysophospholipid;
15	Lme	L-mercptoethanol;
	MAFP	methyl arachidonyl fluorophosphonate;
	mLysoPLA	mouse lysophospholipase;
	PACOCF3	palmitoyl trifluoromethyl ketone;
	PAPO	1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine;
20	PCR	polymerase chain reaction;
	PIP2	phosphatidylinositol 4,5-bisphosphate;
	PIsCho	plasmacytomylin (1-O-(Z)-1 P -alkenyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine);
	TX-100	Triton X-100

25

Definition of Terms

Amino Acid: All amino acid residues identified herein are in the natural L-configuration. In keeping with standard polypeptide nomenclature, J. Biol. Chem., 243:3557-59, (1969), abbreviations for amino acid residues are as shown in the following Table of

30 Correspondence:

TABLE OF CORRESPONDENCE

	<u>SYMBOL</u>	<u>AMINO ACID</u>
<u>1-Letter</u>	<u>3-Letter</u>	
5	Y	Tyr
	G	Gly
	F	Phe
	M	Met
	A	Ala
10	S	Ser
	L	Ile
	L	Leu
	T	Thr
	V	Val
15	P	Pro
	K	Lys
	H	His
	Q	Gln
	E	Glu
20	W	Trp
	R	Arg
	D	Asp
	N	Asn
	C	Cys
25		

It should be noted that all amino acid residue sequences are represented herein by formulae whose left to right orientation is in the conventional direction of amino-terminus to carboxy-terminus. Furthermore, it should be noted that a dash at the beginning or end of an amino acid residue sequence indicates a bond to a radical such as H and OH (hydrogen and hydroxyl) at the amino- and carboxy-termini, respectively, or a further sequence of one or more amino acid residues up to a total of about fifty residues in the polypeptide chain.

Base Pair (bp): A partnership of adenine (A) with thymine (T), or of cytosine (C) with guanine (G) in a double stranded DNA molecule.

35 **Constitutive promoter:** A promoter where the rate of RNA polymerase binding and

initiation is approximately constant and relatively independent of external stimuli. Examples of constitutive promoters include the cauliflower mosaic virus 35S and 19S promoters described by Poszkowski et al., *EMBO J.*, 3:2719 (1989) and Odell et al., *Nature*, 313:810 (1985).

5 **DNA:** Desoxyribonucleic acid.

Enzyme: A protein, polypeptide, peptide RNA molecule, or multimeric protein capable of accelerating or producing by catalytic action some change in a substrate for which it is often specific.

10 **Expression vector:** A DNA sequence that forms control elements that regulate expression of structural genes when operatively linked to those genes.

Expression: The combination of intracellular processes, including transcription and translation undergone by a structural gene to produce a polypeptide.

Insert: A DNA sequence foreign to the rDNA, consisting of a structural gene and optionally additional DNA sequences.

15 **Nucleotide:** A monomeric unit of DNA or RNA consisting of a sugar moiety (pentose), a phosphate, and a nitrogenous heterocyclic base. The base is linked to the sugar moiety via the glycosidic carbon (1' carbon of the pentose) and that combination of base and sugar is a nucleoside. When the nucleoside contains a phosphate group bonded to the 3' or 5' position of the pentose it is referred to as a nucleotide.

20 **Operatively linked or inserted:** A structural gene is covalently bonded in correct reading frame to another DNA (or RNA as appropriate) segment, such as to an expression vector so that the structural gene is under the control of the expression vector.

Polypeptide and peptide: A linear series of amino acid residues connected one to the other by peptide bonds between the alpha-amino and carboxy groups of adjacent residues.

25 **Promoter:** A recognition site on a DNA sequence or group of DNA sequences that provide an expression control element for a gene and to which RNA polymerase specifically binds and initiates RNA synthesis (transcription) of that gene.

Inducible promoter: A promoter where the rate of RNA polymerase binding and initiation is modulated by external stimuli. Such stimuli include light, heat, anaerobic stress, alteration in nutrient conditions, presence or absence of a metabolite, presence of a ligand, microbial attack, wounding and the like.

5 **Spatially regulated promoter:** A promoter where the rate of RNA polymerase binding and initiation is modulated in a specific structure of the organism such as the leaf, stem or root. Examples of spatially regulated promoters are given in Chua et al., *Science*, 244:174-181 (1989).

10 **Spatiotemporally regulated promoter:** A promoter where the rate of RNA polymerase binding and initiation is modulated in a specific structure of the organism at a specific time during development. A typical spatiotemporally regulated promoter is the EPSP synthase-35S promoter described by Chua et al., *Science*, 244:174-181 (1989).

15 **Temporally regulated promoter:** A promoter where the rate of RNA polymerase binding and initiation is modulated at a specific time during development. Examples of temporally regulated promoters are given in Chua et al., *Science*, 244:174-181 (1989).

Protein: A linear series of greater than about 50 amino acid residues connected one to the other as in a polypeptide.

Recombinant DNA molecule: A hybrid DNA sequence comprising at least two nucleotide sequences not normally found together in nature.

20 **RNA:** Ribonucleic acid.

Selective Genetic marker: A DNA sequence coding for a phenotypical trait by means of which transformed cells can be selected from untransformed cells.

Structural gene: A DNA sequence that is expressed as a polypeptide, i.e., an amino acid residue sequence.

25 **Synthetic promoter:** A promoter that was chemically synthesized rather than biologically derived. Usually synthetic promoters incorporate sequence changes that optimize the efficiency of RNA polymerase initiation.

1. Introduction

This invention discloses the cloning, expression and purification of the human LysoPLA (hLysoPLA). The substrate specificity, kinetics and inhibition of pure hLysoPLA was examined. Recently, it was reported that a similar rabbit LysoPLA also functioned as a 5 PLA2 to hydrolyze plasmenylcholine, which is abundant in heart and brain; this is not the case for the human enzyme. Furthermore, the tissue and regional distribution patterns of the enzyme have been characterized herein.

2. Experimental Design, Materials and Methods

10 2.1. Cloning of human *LysoPLA*

Human brain messenger RNA (Clonetech) was used to synthesize first-strand cDNA at 37°C for 1 h using Moloney murine leukemia virus reverse transcripts (Stratagene) and random primers (Promega). Aliquots of the first-strand cDNA were used to amplify the hLysoPLA gene by PCR using Taq DNA polymerase (Promega). The sequences of the 15 primers used in the PCR cycles were: 5 P -GGG GGG CAT ATG TGC GGC AAT AAC ATG TCA ACC CC-3 P and 5 P -GCG CGA ATT CTC AAT CAA TTG GAG GTA GGA GTT TAT-3 P. These primers were designed based on the human sequences found in the GenBank using the mouse LysoPLA sequence as the probe. Restriction enzyme sites (NdeI and EcoRI) were included near the 5 P -ends to facilitate directional cloning (see Fig. 1).

20

The PCR cycles were performed by preheating at 95°C for 1 min, then five cycles of denaturation at 95°C for 45 s, annealing at 60°C for 1 min and extension at 72°C for 2 min. This was followed by 25 cycles of 95°C for 45 s, 66°C for 1 min and 72°C for 2 min. Finally, the PCR product was extended at 72°C for 10 min. The amplified PCR product was about 700 25 bp, and was purified from a 1% agarose gel using Wizard™ PCR Preps (Promega).

To clone the human gene, the purified PCR product was ligated into the pCR2.1

vector (Original TA Cloning Kit from Invitrogen) using T4 DNA ligase (Pharmacia) at 14°C for 19 h. The ligase was heat-inactivated by incubating at 70°C for 10 min. The ligation product was used to transform *Escherichia coli* INVKF P cells, and then plated on LB-Amp (100 µg/ml) with X-Gal. After incubation overnight at 37°C and then 6 h at 4°C, several white colonies were selected for overnight cultures in LB-Amp (100 µg/ml). The plasmids prepared from the over-night cultures were screened by restriction enzyme analysis using *EcoRI* and *NdeI* (Pharmacia). A majority of the screened colonies contained the vector with the correct size insert. The DNA sequence of the insert was obtained using an automated DNA sequencer (Applied Biosystems 373 from Perkin-Elmer). To subclone hLysOPLA from the pCR2.1 vector into the protein expression vector pET28a(+) (Novagen), both the pET28a(+) and the pCR2.1/hLysO-PLA vectors were digested with *NdeI* and *EcoRI*, and then separated on 1% agarose gels. The bands corresponding to hLysOPLA (approx. 700 base pairs) and pET28a(+) (approx. 5300 base pairs) were purified and ligated as described hereinabove. The ligation product was used to transform competent *E. coli* NovaBlue cells (Novagen), and the resulting colonies were screened. It should be noted that the cloned hLysOPLA has an extra 20 amino acids at the N-terminus of the protein, the sequence of which is shown in Sequence 1:

Sequence 1:

As indicated above, the His-Tag can be removed by thrombin cleavage, leaving three extra amino acids at the N-terminus of the protein.

2.2. Expression and purification of hLysoPLA protein

The pET28a(+)hLysoPLA was used to transform competent *E. coli* BL21(DE3) (Novagen), and a single colony was inoculated in an overnight culture in LB-kanamycin (50

μg/ml) medium. This overnight culture was then diluted 40-fold into Terrific broth-kanamycin (50 μg/ml) medium, and allowed to grow at 37°C until the A600 nm reached 1. Then, IPTG (Fisher) was added to a final concentration of 0.4 mM and the cells were grown at 22°C for an additional 5 h to induce foreign protein expression. Finally, the *E. coli* cells 5 were centrifuged and the pellet was stored at 32°C. The purification procedures were carried out at 4°C, as described previously.

Briefly, the *E. coli* pellet was resuspended in lysis bu~~jer~~ (25 mM Tris- HCl, pH 8.0, 500 mM NaCl, 5 mM imidazole and 10 mM LMe) and digested by lysozyme (Sigma). The 10 mixture was sonicated and centrifuged at 100,000 U g for 45 min. The supernatant was passed through a Ni-NTA column (Qiagen), the column washed with lysis bu~~jer~~, and the protein eluted with elution buffer (25 mM Tris-HCl, pH 8.0, 500 mM NaCl, 250 mM imidazole and 10 mM LMe). The eluted hLysoPLA was then loaded onto a Sephadex G-75 (2.5 U 90 cm, 15 Pharmacia) column equilibrated in buffer containing 10 mM each of Tris-HCl (pH 8.0), NaCl and LMe. The hLysoPLA that eluted from the G-75 column was essentially homogeneous. The His W Tag was removed by digestion with biotinylated thrombin (Novagen) overnight at 4°C. The biotinylated thrombin was removed at the end of the digestion by streptavidin 20 agarose (Novagen), and the cleaved His W Tag removed by dialysis against 10 mM each of Tris (pH 8.0) and LMe. The enzyme was stored at 32°C after glycerol was added to a final concentration of 50% and LMe to 10 mM.

2.3. Northern blot

The 32 P-labeled probe was prepared by incubating 50 ng cDNA of hLysoPLA with 50 μCi [32 P]dCTP (DuPont-NEN) and Ready-To-Go DNA labeling beads (-dCTP) 25 (Pharmacia Biotech) at 37°C for 15 min, as described by the protocol supplied by the manufacturer. The 32 P-labeled cDNA probe was purified through a ProbeQuant G-50 microcolumn (Pharmacia Biotech). The human RNA blot (Clone- tech) was prehybridized

with 20 ml of ExpressHyb solution (Clonetech, CA) containing 1 mg/ml salmon sperm DNA at 65°C for 30 min. The RNA blot was hybridized with 5 ml of ExpressHyb containing 6 μ g/ml of human Cot-1 DNA, 30 μ g/ml of salmon sperm DNA, 0.2 U SSC (sodium chloride and sodium citrate) and 10⁷ cpm of human lysophospholipase cDNA probe (with specific activity of 1.5 U 10⁹ cpm/ μ g) overnight at 65°C. After hybridization, the membrane was washed once with 200 ml of 2 U SSC containing 1% SDS at 65°C for 20 min, and twice with 0.1 U SSC containing 0.5% SDS at 55°C for 20 min each. The membrane was exposed to X-ray film for 24 h at 37°C. After developing, the film was read with a densitometer and each dot was quantitated.

10

2.4. Western blot

The tissue distribution pattern of hLysoPLA at the protein level was examined by Western blot analysis using rabbit antibody (customer-made by HTI BioProducts) raised against purified recombinant hLyso- PLA. First, 70 μ g of protein from different human tissues (Clonetech) were separated by 12% SDS- PAGE along with prestained protein molecular weight markers (Bio-Rad) and pure hLysoPLA. The proteins in the gel were then transferred to a PVDF membrane (Millipore). To block any non-specific binding, the membrane was incubated first in 5% non-fat milk in PBS (phosphate-buffered saline), followed by rabbit pre-immune serum (1/1000 dilution with PBS containing 0.5% Tween 20 and 0.5% milk) for 1 h. The protein bands recognized by the preimmune serum were visualized and blocked by treating the membrane with horseradish peroxidase-conjugated protein A (Amersham) and VECTOR SG substrate kit for peroxidase (Vector Laboratories). Then, the membrane was probed with the rabbit anti-hLysoPLA serum (1/1000 dilution) for 1 h, followed by horseradish peroxidase-conjugated protein A (Amersham). Finally, the protein bands were detected by the ECL system (Amersham).

2.5. *LysoPLA activity, PLA2 activity and protein purity determination*

The protein concentration was determined by the Bio-Rad protein assay, using bovine serum albumin (BSA) as standard. Protein purity was examined using 12% SDS-PAGE and Coomassie blue staining. LysoPLA activity was measured at 40°C in the assay buffer containing LysoPC, 100 mM Tris-HCl (pH 8.0) and 10 mM LMe. Cold 1-palmitoyl-sn-glycero-3-phosphorylcholine (125 µM, unless indicated otherwise) was mixed with 1-[¹⁴C]palmitoyl-sn-glycero-3-phosphorylcholine (100,000 cpm) in a total volume of 0.5 ml. The assay was initiated by adding an aliquot of enzyme solution to the substrate mixture and incubating for the desired time. The released fatty acid was extracted by the modified Dole method and then quantified by scintillation counting. The PLA2 activity assay was carried out, using various ¹⁴C-labeled phospholipid substrates under various conditions as given in the text. The rat brain iPLA2 was purified as described elsewhere (H.C. Yang et al., manuscript in preparation incorporated herein). Human recombinant cPLA2 was a gift from Drs. J.D. Sharp and R.M. Kramer at Lilly Laboratories. 1-[¹⁴C]Palmitoyl-sn-glycero-3-phosphorylcholine and 1-palmitoyl-2-[1-¹⁴C]palmitoyl-sn-glycerol-3-phosphorylcholine were purchased from Amersham. 1-Palmitoyl-2-[1-¹⁴C]arachidonoyl-sn-glycerol-3-phosphorylcholine was purchased from DuPont-NEN. All unlabeled phospholipids were purchased from Avanti Polar Lipids. Triton X-100 (TX-100) and PIP2 were obtained from CalBiochem.

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2.6. *Synthesis of plasmenylcholine*

The sn-2 labeled PlsCho (plasmenylcholine or 1-O- (Z)-1 P -alkenyl-2-arachidonoyl-sn-glycero-3-phosphocholine) was synthesized as follows. A total of 4 µmol [1-¹⁴C]arachidonic acid (from NEN, with specific activity 63.3 µCi/µmol) was diluted 10 times with cold arachidonic acid in 250 µl distilled methylene chloride (Fisher Scientific). To this 0.04 mmol p(N,N-dimethyl)amino pyridine (Aldrich) and 0.08 mmol 1,3-dicyclohexyl carbodiimide (Aldrich) were added to yield the arachidonoyl anhydride upon 1 h of stirring.

Lysoplasmenylcholine (0.06 mmol, from Serdary) dissolved in 200 μ l methylene chloride and 50 μ l pyridine (Fisher Scientific) was added to the arachidonoyl anhydride solution with 0.03 μ mol dimethylaminopyridine and left stirring for 8 h. The reaction progress was monitored by thin layer chromatography (Merck) using a mobile phase of chloroform:methanol:triethyl
5 amine:water (65:30: 1.5:3.5), and retardation factors were 0.6 and 0.2 for PlsCho and LysoPlsCho, respectively. The separation of the product was carried out by preparative layer chromatography (Merck) in the above mobile system. The reaction yielded a total of 3.6 mg (7.7 μ mol) of radiolabeled PlsCho (specific activity 0.18 μ Ci/ μ mol) by quantitative phosphate analysis.

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2.7. Inhibition studies of hLysoPLA

Various concentrations of each inhibitor (BioMol) were prepared in DMSO. 5 μ l of each inhibitor was added to 480 μ l of LysoPC substrate (125 μ M Ly- soPC in the assay buffer). Enzyme (either mouse or human PLA) was added to the substrate-inhibitor mixture to
15 start the reaction, which was incubated at 40°C for 30 min. Control experiments were performed with the same amount of enzyme with 5 μ l DMSO in the incubation mixture. The reversibility of the inhibition was tested by pre-incubation of the enzyme in the presence or absence of the inhibitor, followed by overnight dialysis in the assay buffer. The recovery of the enzyme activity as a function of time was then followed. Each data point represents an
20 average of duplicates.

EXAMPLE 1.

Cloning and expression of hLysoPLA.

LysoPLA plays important roles in regulating the level of the multifunctional LysoPL, and several lines of evidence have suggested that LysoPLA exists in human. First, a relatively high level of LysoPLA activity has been found in human brain and it was proposed to be the major route by which LysoPL is removed from cell membranes [43]. Second, two human
25

LysoPLAs (molecular masses of 20 and 22 kDa) have been purified from human HL-60 cells [55]. These two enzymes appear to be the human isoforms of the mouse 27 and 28 kDa LysoPLAs. Third, when we searched DNA and protein databases in GenBank using mouse LysoPLA sequence information, several human cDNA clones were identified that match to 5 different parts of the mouse gene Wang et al. / Biochimica et Biophysica Acta 1437 (1999) 157^169 with high homology. However, these cDNA sequences were obtained from different human tissues, their functions not identified, and none of them contained the entire protein sequence.

10 To determine the sequence of the human LysoPLA and to study the properties and functions of this enzyme in lipid metabolism and signal transduction, we have cloned this hLysoPLA using RT-PCR techniques. Normal human brain mRNA was used to generate the first-strand cDNA, from which a 700 bp product was amplified using primers corresponding to the N and C-terminals of hLysoPLA (Fig. 1). Then, the 700 bp DNA was ligated to the 15 pCR2.1 vector and sequenced, as shown in Fig. 1 along with the translated protein sequences. The hLysoPLA is a single polypeptide composed of 230 amino acids with a calculated molecular mass of 25 kDa. The hLysoPLA sequence is highly homologous to the mouse/rat LysoPLA, 89% on the DNA level, and 92% on the protein level. The altered nucleotides and amino acids (shown in bold in Fig. 1) are spread out over the entire sequence, probably due to 20 the random mutations of an ancestor enzyme during evolution. The Ser-119, Asp-174 and His-208 triad that forms the catalytic site for the mLysoPLA [46] is preserved in the human enzyme, indicating that the catalytic mechanism is also conserved.

25 To express hLysoPLA, *E. coli* transformed with the protein expression vector pET28a(+)/hLysoPLA was induced with 0.4 mM IPTG. SDS-PAGE analysis of cell homogenates showed that a 28 kDa protein band is strongly induced (lane 2 vs. lane 1 in Fig. 2). The recombinant protein was purified in two steps. The first was Ni-NTA chromatography

that removed the vast majority of the contamination proteins, and the second was G-75 chromatography that removed the minor high molecular weight contamination and exchanged the enzyme into a low salt buffer in which it is more stable. The hLysoPLA thus obtained was homogeneous as estimated by the SDS-PAGE (lane 3 in Fig. 2). The His-Tag at the N terminus of the protein was removed by thrombin, as shown in lane 4 of Fig. 2. The recombinant hLysoPLA thus obtained has an extra three amino acid residues (namely, Gly-Ser-His) at the N terminus. The specific activity of the recombinant hLysoPLA is about 1.3 μ mol/min/mg toward palmitoyl LysoPC, which is the same as the value reported for the 27 kDa mLysoPLA. This demonstrates that the sequence shown in Fig. 1 does indeed encode an active human LysoPLA.

EXAMPLE 2

Kinetic characterization of the recombinant hLysoPLA

To investigate the catalytic properties of the hLysoPLA, we first determined the specific activity of hLysoPLA as a function of substrate (1-palmitoyl LysoPC) concentration. As shown in Fig. 3, the catalytic activity exhibits saturation kinetics. However, the data did not fit well to the Michaelis-Menten equation (dotted line in Fig. 3). There seems to be some cooperativity, since the data fit to the Hill equation (solid line in Fig. 3) with $n = 1.53 \pm 0.08$, $k_{cat} = 1.34 \pm 0.04 \mu$ mol/min/mg, and substrate at half-maximal saturation is $12.7 \pm 0.9 \mu$ M. Furthermore, there is no dramatic increase in the enzyme activity near the cmc of the substrate (7 μ M), indicating that the enzyme hydrolyzes both monomeric and micellar substrates. This suggests that hLysoPLA is not an interfacially activated enzyme.

TX-100 is often used in the purification and stabilization of membrane-associated proteins. In addition, it is used to change the surface concentration of the substrate in the mixed-micelle assay for mechanistic studies [56,57]. To determine the dependence of the enzyme activity on the substrate surface concentration, we measured the hLysoPLA activity

under two conditions:

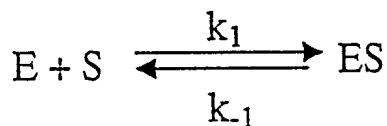
(1) the bulk LysoPC concentration (1.125 mM) was kept constant and the TX- 100 concentration was varied (Fig. 4A);

5 (2) the bulk TX-100 concentration (1 mM) was kept constant and the LysoPC concentration was varied (Fig. 4B).

In both cases, enzyme activity increases as the percentage of LysoPC substrate in the micelles increases, and both sets of data fit well to the Michaelis-Menten equation with similar values of k_{cat} (1.6 $\mu\text{mol}/\text{min}/\text{mg}$ and 1.7 $\mu\text{mol}/\text{min}/\text{mg}$). These k_{cat} values are also 10 very close to that obtained in Fig. 3, a result expected if TX-100 serves as a neutral surface diluter of the substrate.

In addition, we have carried out detailed kinetic measurements and analysis to test the surface dilution kinetic model and results are shown in Fig. 4°C. The experimental data were 15 fitted globally to Eq. 1 derived from the model in which hLysoPLA follows the two-step binding kinetics (Scheme 1). That is, hLysoPLA (E) binds first to the mixed-micellar surface (S) and then to the LysoPC substrate (A) presented on the surface.

Scheme 1:



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Equation 1:

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$$v = \frac{V_{max} [LysoPC + TX-100] \{LysoPC\}}{K_m K_s + K_m [LysoPC + TX-100] + [LysoPC + TX-100] \{LysoPC\}}$$

Here, $[LysoPC+TX-100]$ is the total molar concentration in the bulk solution, and $\{LysoPC\}$ is the molar fraction of the substrate on the mixed-micellar surface. Calculation of the amount of TX-100 in the micelle takes into account the amount of monomeric detergent in the solution, assuming the critical micelle concentration of TX-100 is 0.25 mM. The fitting parameter K_s represents the dissociation constant (k_{31}/k_1) for the mixed-micellar surface, K_m represents the apparent Michaelis constant ($=(k_3+k_{32})/k_2$) on the surface, and V_{max} is the catalytic turnover rate. The values of parameters obtained from global fits are: $V_{max} = 1.37 \pm 0.05 \mu\text{mol}/\text{min}/\text{mg}$, $K_m = 12.2 \pm 0.9 \text{ mol\%}$ and $K_s = 66 \pm 7 \mu\text{M}$. These data are consistent with the following three conclusions.

10

First, the fact that a single value for K_s fits all the experimental data suggests that this enzyme binds to mixed-micelles with the same affinity regardless of the micellar composition. Thus, the first binding step is likely to occur through a non-specific hydrophobic interaction with the surface. Second, the enzyme appears to act primarily on the substrate within the interface. This conclusion is also supported by surface dilution kinetics observed in Fig. 4A and 4B. Third, the dependence of apparent V_{max} on mol% of LysoPC in the micelle is consistent with a single binding site for the substrate. This 1:1 stoichiometry of substrate-enzyme complex suggests that the apparent cooperativity seen in the kinetics of hLysoPLA on pure LysoPC micelles (Fig. 3) results from the change in the substrate presentation (monomers vs. premicellar aggregates vs. micelles) rather than a cooperative binding of two or more substrates to catalytic or allosteric sites.

EXAMPLE 3

25 ***Insignificant PLA2 activity of hLysoPLA***

When we first purified the mouse LysoPLA from P388D1 cells, no other enzymatic activities (PLA1, PLA2, acyltransferase, transacylase and esterase) were found. Therefore, it

appears to be a LysoPL-specific LysoPLA. Similar results were obtained for the rat LysoPLA and the relative rates towards different LysoPL substrates were reported [Sugimoto et al., (1996) J. Biol. Chem. 271, 7705-11]. However, a recently sequenced rabbit enzyme that shares high sequence homology (approx. 97%) to the rat/ mouse LysoPLA was reported 5 to have both PLA2 and LysoPLA activities [Portilla, et al., (1968) J.Am.Soc.Nephrol. 9, 1178-86]. The specific activities, however, were only 10 nmol/min/mg for PLA2 activity and 20 nmol/min/mg for LysoPLA activity. The PLA2 activity was also reported to be selective for PlsCho substrate with arachidonic acid at the sn2 position. To determine whether hLysoPLA also expresses PLA2 activity, we have explored its ability to hydrolyze 10 arachidonic acid labeled PlsCho as well as other phospholipid substrates under a variety of conditions. As summarized in Table 1, hLysoPLA did not show any significant activity toward the PlsCho substrate labeled with [14 C]arachidonic acid, whether in mixed micelles or in vesicles. Also no PLA2 activity was found for hLysoPLA toward several phosphatidylcholine substrates.

15 As a control, LysoPLA activity toward LysoPC was determined at the same time with the same batch of enzyme. Since the hLysoPLA was active toward LysoPC, the insignificant PLA2 activity of hLysoPLA is not due to the inactivation of the enzyme. In comparison, cPLA2 (an arachidonic acid-selective PLA2) showed a relatively high activity toward PlsCho 20 when the substrate was presented in the presence of 3 mol% PIP2, which is about 60% of its activity toward PAPC under the same conditions. Rat brain iPLA2 demonstrated a weak but significant activity toward PlsCho. It is 10- and 4-fold lower than its activity toward DPPC and PAPC TX-100 mixed micelles, respectively.

25 Because hLysoPLA did not show any significant activity toward any diradylglycerophospholipids (PlsCho, PAPC, DPPC) under all the conditions tested, the hLysoPLA is not a PLA2. In light of the high sequence homology among the mouse, rat and

human LysoPLAs, and the fact that these enzymes exhibited the same kinetic properties, we propose that these LysoPLAs, as a group, are specific LysoPLAs without other significant enzymatic activities. The rabbit enzyme was originally reported to be only a PLA2 without any significant LysoPLA activity [Portilla et al., (1996) J. Biol. Chem. 271, 15451-57], and 5 only recently was the LysoPLA activity found after the enzyme showed high sequence homology to the rat/mouse LysoPLA. The specific activity of the rabbit enzyme as a LysoPLA, however, was more than 50-fold lower than the specific activity of the corresponding mouse, rat and human enzymes under similar assay conditions. We have found several factors that can reduce the LysoPLA activity significantly and may contribute to the 10 lower LysoPLA observed for the rabbit enzyme. For example, LysoPLA appears to be quite unstable and the temperature/solution components of the enzyme during purification, storage and assay affect its activity substantially. The percentage of hydrolysis and linearity of the reaction also need to be considered when the activity is determined from a single time point measurement. Because of the sequence homology between the rabbit enzyme and the human, 15 rat and mouse LysoPLAs, the rabbit enzyme may be a LysoPL-specific LysoPLA as well.

EXAMPLE 4

Inhibition of the LysoPLA

Several small organic compounds were examined for their abilities to inhibit the 20 mouse and human LysoPLA, and similar results were found for both enzymes. PACOCF3 and BEL are iPLA2 inhibitors, but neither of them inhibited the LysoPLA activity well. LysoPAF, a LysoPC substrate analog with an ether linkage in the sn-1 position, was not a good inhibitor either. MAFP is an inhibitor of both iPLA2 and cPLA2. Here, we found that it also inhibited LysoPLA with an apparent IC₅₀ of 0.6 μ M (Fig. 5). The inhibition of LysoPLA 25 by MAFP is irreversible, since LysoPLA remained inactive when the enzyme and MAFP mixture was assayed after overnight dialysis. The inhibition of LysoPLA by MAFP is most likely due to the covalent modification of the enzyme at the active site Ser-119. MAFP has

Table I. Substrate selectivity of lysophospholipase compared with cPLA₂ and iPLA₂ toward plasmenylphosphatidylcholine and phosphatidylcholine under various conditions. As a control, LysoPLA activity toward the LysoPC substrate was also included.

Enzyme	Substrate	Assay conditions	Activity ($\mu\text{mol}/\text{min}/\text{mg}$)
Human Recombinant LysoPLA	LysoPC micelles (125 μM)	100 mM Tris, pH 8.0, 10 mM βMe	1.13 \pm 0.03
	PIsCho/TX-100 mixed micelles (100 μM : 600 μM)	100 mM Hepes, pH 7.5, 10 mM βMe	0.0024 \pm 0.0004
	PIsCho sonicated vesicles (100 μM)	100 mM Tris, pH 8.0, 4 mM EGTA, 10 mM βMe	0.0009 \pm 0.0003
	PIsCho sonicated vesicles (100 μM)	100 mM Tris, pH 8.0, 150 mM NaCl, 3 mM MgCl_2 , 10 mM βMe	0.0006 \pm 0.0002
	DPPC/TX-100 mixed micelles (100 μM : 400 μM)	100 mM Hepes, pH 7.5, 2 mM DTT, 5 mM EDTA	<0.0001
	DPPC/POPS sonicated vesicles (67 μM : 33 μM)	100 mM Hepes, pH 7.5, 5 mM EDTA or Ca^{2+} , 1 mg/ml BSA	<0.0001
	PAPC/TX-100 mixed micelles (100 μM : 400 μM)	100 mM Hepes, pH 7.5, 80 μM Ca^{2+} , 2 mM DTT, 0.1 mg/ml BSA	<0.0001
	PAPC/PIP ₂ /TX-100 mixed micelles (97 μM : 3 μM : 400 μM)	100 mM Hepes, pH 7.5, 80 μM Ca^{2+} , 2 mM DTT, 0.1 mg/ml BSA	<0.0001
Human Recombinant cPLA ₂	PIsCho/PIP ₂ /TX-100 mixed micelles (100 μM : 3 μM : 600 μM)	100 mM Hepes, pH 7.5, 80 μM Ca^{2+} , 0.1 mg/ml BSA, 2 mM DTT.	13.58 \pm 0.05
	PAPC/PIP ₂ /TX-100 mixed micelles (97 μM : 3 μM : 400 μM)	100 mM Hepes, pH 7.5, 80 μM Ca^{2+} , 0.1 mg/ml BSA, 2 mM DTT.	21.81 \pm 0.09
Rat brain iPLA ₂	PIsCho/TX-100 mixed micelles (100 μM : 600 μM)	100 mM Hepes, pH 7.5, 5 mM EDTA, 2 mM DTT	0.53 \pm 0.03
	DPPC/TX-100 mixed micelles (100 μM : 400 μM)	100 mM Hepes, pH 7.5, 5 mM EDTA, 2 mM DTT	5.6 \pm 0.2
	PAPC/TX-100 mixed micelles (100 μM : 400 μM)	100 mM Hepes, pH 7.5, 5 mM EDTA, 2 mM DTT	2.0 \pm 0.04

been used in signal transduction studies as a selective cPLA₂ inhibitor. However, as it also inhibits both LysoPLA and iPLA₂ activities, the contribution of each of these enzymes to signal transduction should be more carefully considered.

5

EXAMPLE 5

Tissue distribution of hLysoPLA

To examine the tissue distribution pattern of LysoPLA, we have carried out Northern and Western blots using either the human LysoPLA gene as a Northern blot probe, or the rabbit antibody raised against pure recombinant hLysoPLA. As shown in Fig. 6A, the size of

10 hLysoPLA messenger is about 2.4 kb. The mRNA is widely distributed in many tissues, with heart, placenta and skeletal muscle being the most abundant ones, followed by liver, pancreas, kidney, brain and lung. On the Northern master blot which has messengers from more tissues (Fig. 6B,C), similar patterns were observed though the relative intensity for a few tissues was changed compared to Fig. 6A. In Fig. 6B,C, placenta and testis are the most abundant sources for hLysoPLA, followed by adrenal and salivary glands, liver, heart, skeletal muscle, and trachea colon. In contrast to the mRNA levels in adult tissues, the fetal mRNA of hLysoPLA appears to be much more uniform among different tissues. To screen the expression of hLysoPLA in different tissues, Western analysis was performed with rabbit polyclonal antibody for the hLysoPLA. As shown in Fig. 6D, testis expressed the most abundant hLysoPLA, followed by kidney, brain, placenta, liver, heart and ovary.

DISCUSSION

These examples define a new, cloned, expressed and characterized human lysophospholipid-specific LysoPLA. The enzyme is widely distributed in almost all tissues at both mRNA and protein levels, although the relative amount varies greatly from tissue to tissue. The human LysoPLA is homologous to the rat, mouse and rabbit enzymes, with the catalytic triad conserved in all and the changed residues spread randomly along the protein

sequences. The mouse and human enzymes exhibit similar inhibition patterns toward various inhibitors, with MAFP being the most potent one. Detailed kinetic analysis shows that the human LysoPLA displays apparent co-operativity and surface dilution kinetics. The enzyme appears to bind nonspecifically to the mixed-micellar surface first, followed by the specific binding to the substrate on the surface.

The kinetic parameters associated with this surface dilution kinetic model were determined, and it appears that hLysoPLA has a single substrate binding site and a surface nonspecific recognition site. The apparent co-operativity is likely due to the change of substrate presentation. In addition, the hLysoPLA did not display any significant PLA2 activity toward PlsCho and phosphatidylcholine in both mixed-micelle and vesicle forms. Since no significant PLA1, PLA2, acyltransferase, transacylase and esterase activities were found for rat and mouse LysoPLA, we conclude that these enzymes form a group of specific LysoPLAs that recognize and act on only lysophospholipids.

Lysophospholipases are critical enzymes that act on biological membranes to regulate the multifunctional lysophospholipids; increased levels of lysophospholipids are associated with a host of diseases. The instant invention describes the cDNA cloning of a human brain 25 kDa lysophospholipid-specific lysophospholipase (hLysoPLA).

The enzyme (at both mRNA and protein levels) is widely distributed in tissues, but with quite different abundances. The hLysoPLA hydrolyzes lysophosphatidylcholine in both monomeric and micellar forms, and exhibits apparent cooperativity and surface dilution kinetics, but not interfacial activation. Detailed kinetic analysis indicates that the hLysoPLA binds first to the micellar surface and then to the substrate presented on the surface. The kinetic parameters associated with this surface dilution kinetic model are reported, and it is concluded that hLysoPLA has a single substrate binding site and a surface recognition site.

The apparent cooperativity observed is likely due to the change of substrate presentation. In contrast to many nonspecific lipolytic enzymes that exhibit lysophospholipase activity, hLysoPLA hydrolyzes only lysophospholipids and has no other significant enzymatic activity. Of special interest, hLysoPLA does not act on plasmenylcholine. Of the several inhibitors 5 tested, only methyl arachidonyl fluorophosphonate (MAFP) potently and irreversibly inhibits the enzymatic activity. The inhibition by MAFP is consistent with the catalytic mechanism proposed for the enzyme - a serine hydrolase with a catalytic triad composed of Ser-119, Asp-174 and His-208.

10 While the present invention has now been described in terms of certain preferred embodiments, and exemplified with respect thereto, one skilled in the art will readily appreciate that various modifications, changes, omissions and substitutions may be made without departing from the spirit thereof. It is intended, therefore, that the present invention be limited solely by the scope of the following claims.

15

CLAIMS

What is claimed is:

1. A cDNA segment encoding a lysophospholipid-specific human brain lysophospholipase enzyme protein molecule, comprising:
 - 5 a segment of 690 deoxyribonucleotide base pairs from position 448 to position 741 in Figure 1.
2. A deoxyribonucleic acid sequence for a cDNA segment encoding a lysophospholipid-specific human brain lysophospholipase enzyme protein molecule, comprising:
 - 10 a sequence of 690 deoxyribonucleotide base pairs from position 448 to position 741 in Figure 1.
3. A recombinant lysophospholipid-specific human brain lysophospholipase enzyme protein molecule, comprising:
 - 15 a single 25 kDa polypeptide having 230 amino acid residues; and a catalytic activity site triad at Ser-119, Asp-174 and His-208.
4. An amino acid residue sequence for a recombinant lysophospholipid-specific human brain lysophospholipase enzyme protein molecule, comprising:
 - 20 amino acid residues from amino acid residue position 1 to amino acid residue position 230 in Figure 1.
5. A method for inhibiting catalytic activity of a recombinant lysophospholipid-specific human brain lysophospholipase enzyme protein molecule, comprising:

exposing the lysophospholipase enzyme protein molecule to a solution containing methyl arachidonyl fluorophosphates.

6. The method for inhibiting catalytic activity according to claim 5, wherein the inhibiting resulting from the exposing is irreversible.

5

7. A method for treating patients with diseases caused by increased levels of lysophospholipids, comprising:

Supplying a recombinant lysophospholipid-specific lysophospholipase enzyme to an enzyme deficient patient.

10

8. The method for treating patients according to claim 7, wherein the supplying transpires by infusion.

15

9. The method for treating patients according to claim 7, wherein the supplying transpires by gene augmentation therapy.

20

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ABSTRACT

A cloned of a human brain lysophospholipid-specific lysophospholipase enzyme molecule, its potential use for treatment of a host of diseases and method of inactivation are disclosed. Also disclosed are its distribution in tissue sand detailed kinetic analysis. hLysoPLA has a single substrate binding site and a surface recognition site. In contrast to many nonspecific lipolytic enzymes that exhibit lysophospholipase activity, hLysoPLA hydrolyzes only lysophospholipids and has no other significant enzymatic activity.

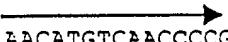
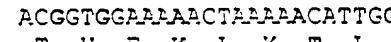
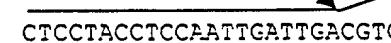
cDNA	CCGGGCGCCCGCTTCCCGCTTGCCTGTGAGCTGAGGGGTATGTGCGGCAAT	60
amino acid	M C G N	4
cDNA	 AACATGTCAACCCCGCTGCCGCCATCGTCCCCGCCGGAAAGGCCACCGCTGCCGTG	120
amino acid	N M S T P L P A I V P A A R K A T A A V	24
cDNA	ATTTTCTGCATGGATTGGAGATACTGGCACGGATGGCAGAAGCCTTGCAGGTATC	180
amino acid	I F L H G L G D T G H G W A E A F A G I	44
cDNA	AGAAGTTCACATATCAAATATATCTGCCCGCATGCCCTGTTAGGCCTTACATTAAAT	241
amino acid	R S S H I K Y I C P H A P V R P V T L N	64
cDNA	ATGAAACGTGGCTATGCCCTCATGGTTGATATTATTGGGCTTCACCAAGATTACAGGAG	300
amino acid	M N V A M P S W F D I I G L S P D S Q E	84
cDNA	GATGAATCTGGGATTAAACAGGCAGCAGAAAAATAAAAGCTTGATTGATCAAGAAGTG	360
amino acid	D E S G I K Q A A E N I K A L I D Q E V	104
cDNA	AAGAATGGCATTCCCTCTAACAGAATTATTTGGGAGGGTTCTCAGGGAGGAGCTTA	420
amino acid	K N G I P S N R I I L G G F S Q G G A L	124
cDNA	 TCTTTATATACTGCCCTTACCAACACAGCAGAAACTGGCAGGTGTCACTGCACTCAGTTGC	480
amino acid	S L Y T A L T T Q Q K L A G V T A L S C	144
cDNA	TGGCTTCCACTTCGGGCTTCCCTTCCACAGGGTCTATCGGTGGTGTAAATAGAGATATT	540
amino acid	W L P L R A S L P Q G P I G G A N R D I	164
cDNA	TCTATTCTCCAGTCCCACGGGGATTGTGACCCCTTGGTCCCTGATGTTGGTTCTTT	600
amino acid	S I L Q C H G D C D P L V P L M F G S L	184
cDNA	 ACGGTGGAATAACTAAAAACATTGGTAATCCAGCCAATGTGACCTTAAACCTATGAA	660
amino acid	T V E K L K T L V N P A N V T F K T Y E	204
cDNA	 GGTATGATGCACAGTTGTCAACAGGAAATGATGGATGTCAAGCAATTGATAAA	720
amino acid	G M M H S S C Q Q E M M D V K Q F I D K	224
cDNA	 * EcoRI CTCCTACCTCCAATTGATTGACGTCACTAAGAGGCCCTT	758
amino acid	L L P P I D *	230

Fig 1

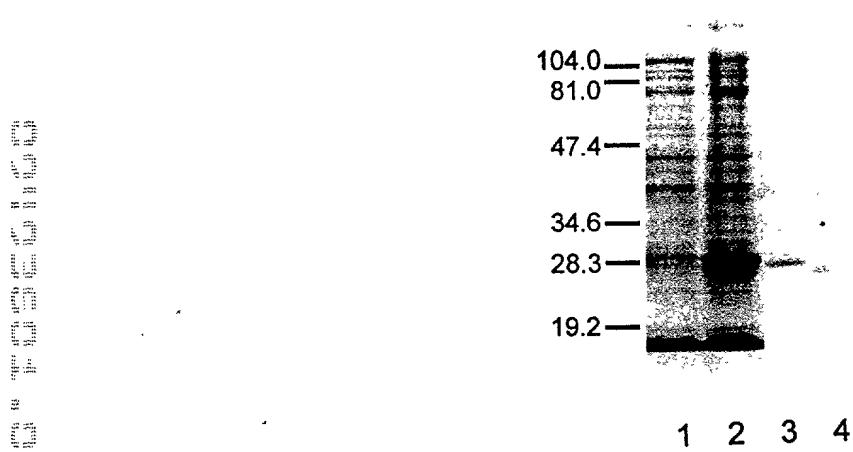


Fig. 2.

SPECIFIC ACTIVITY
(nmol/min/mg)

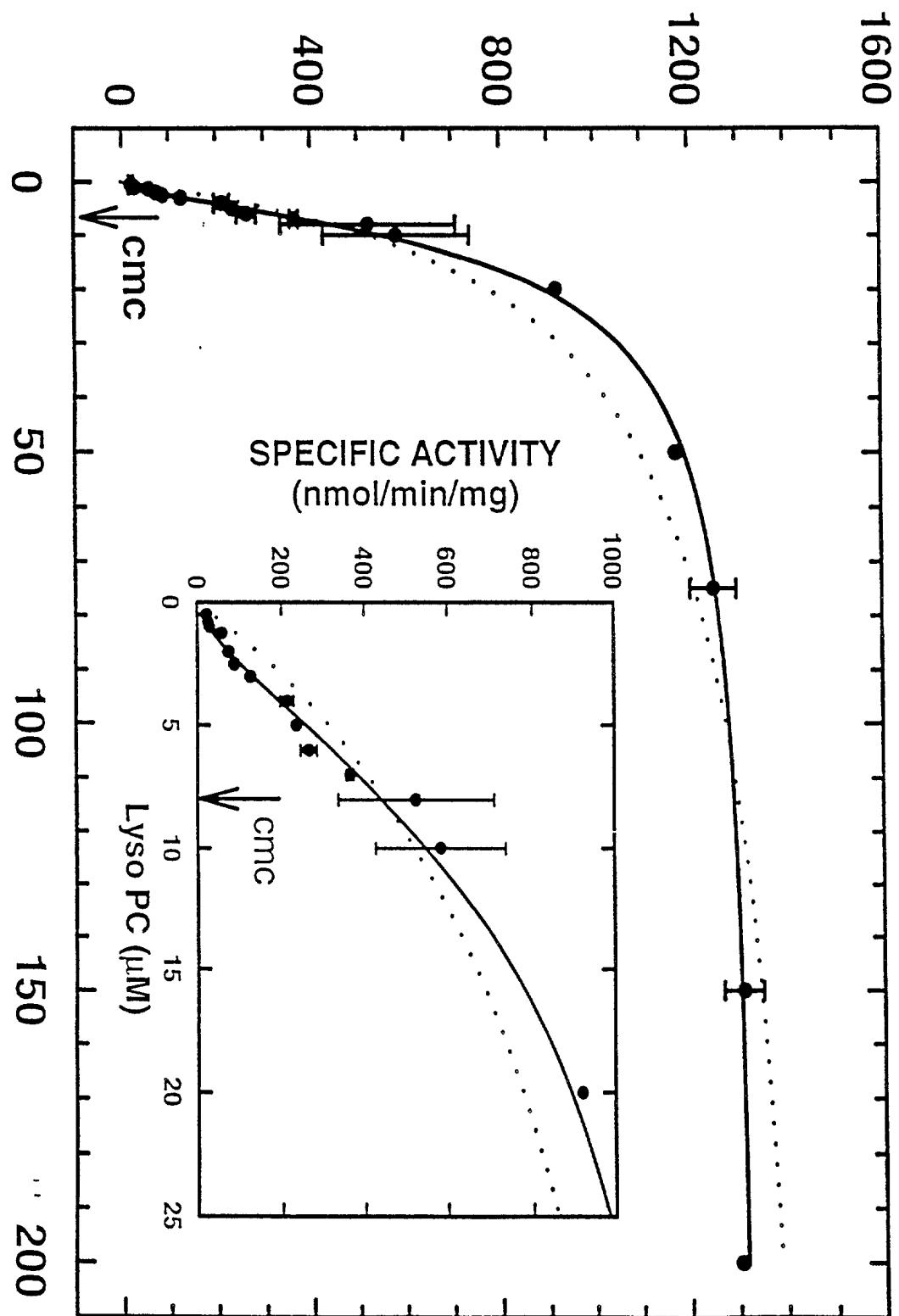


Fig 3

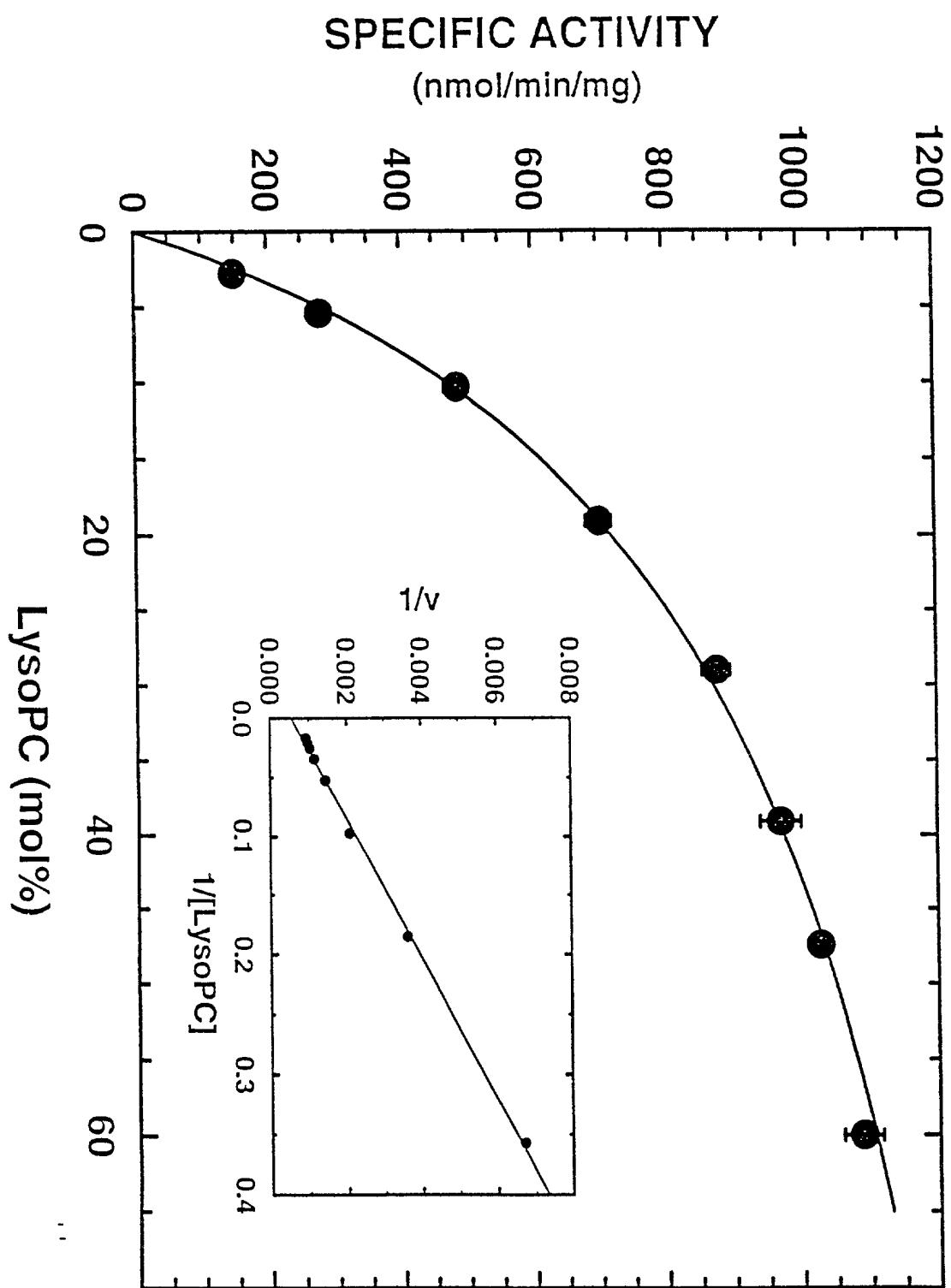
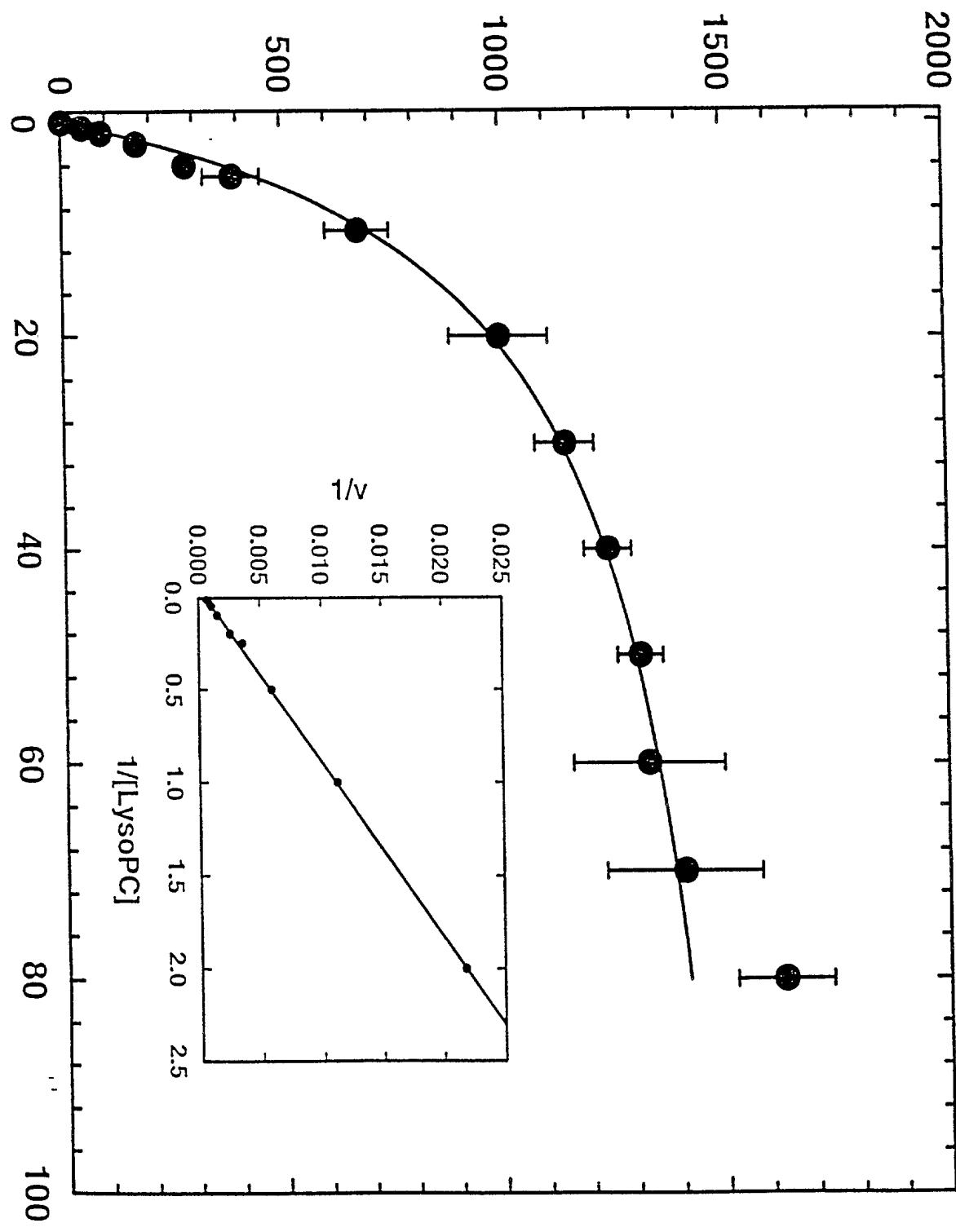


Fig 4A

SPECIFIC ACTIVITY

(nmol/min/mg)



LysoPC (mol%)

Fig 4B

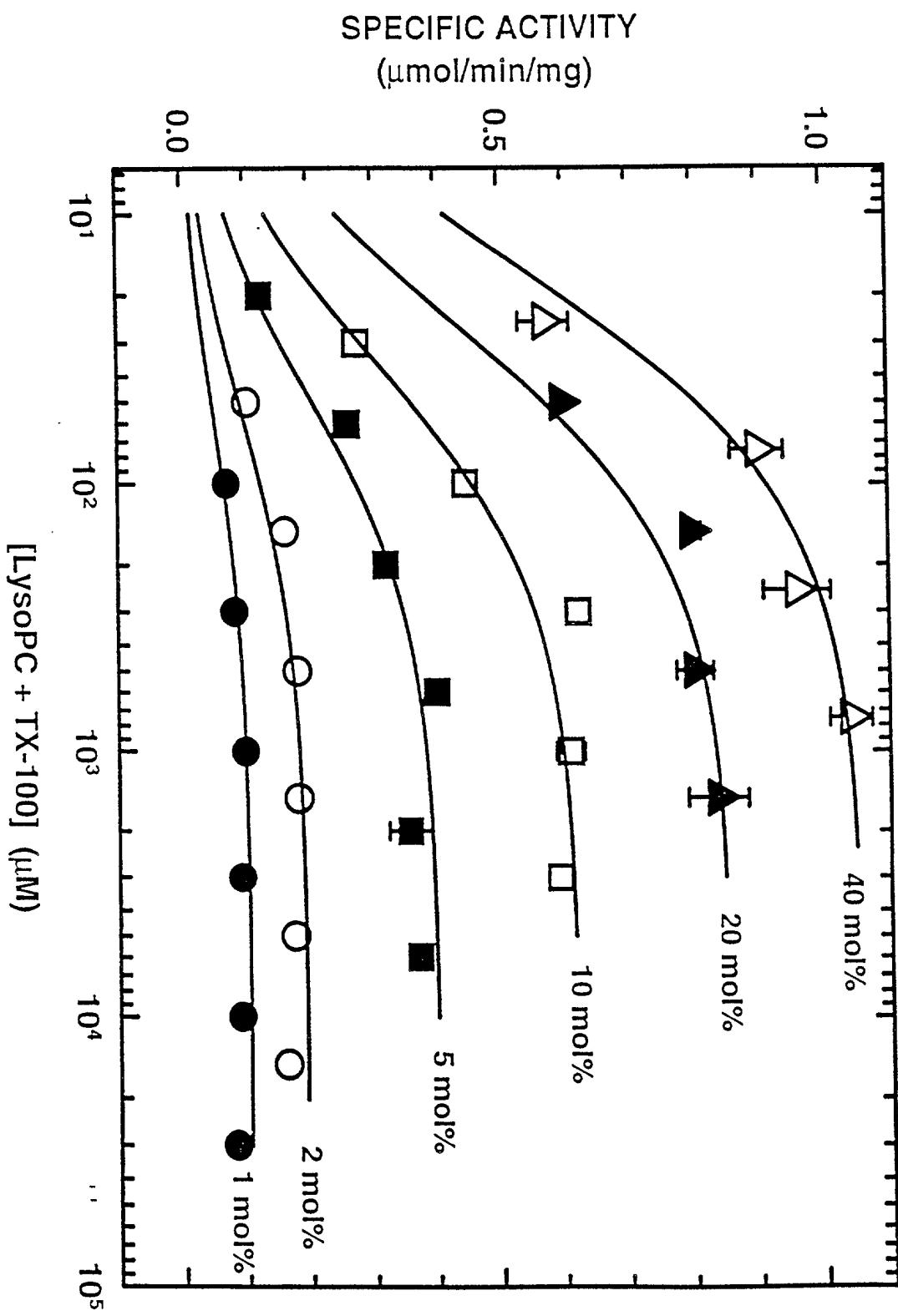


FIG 4C

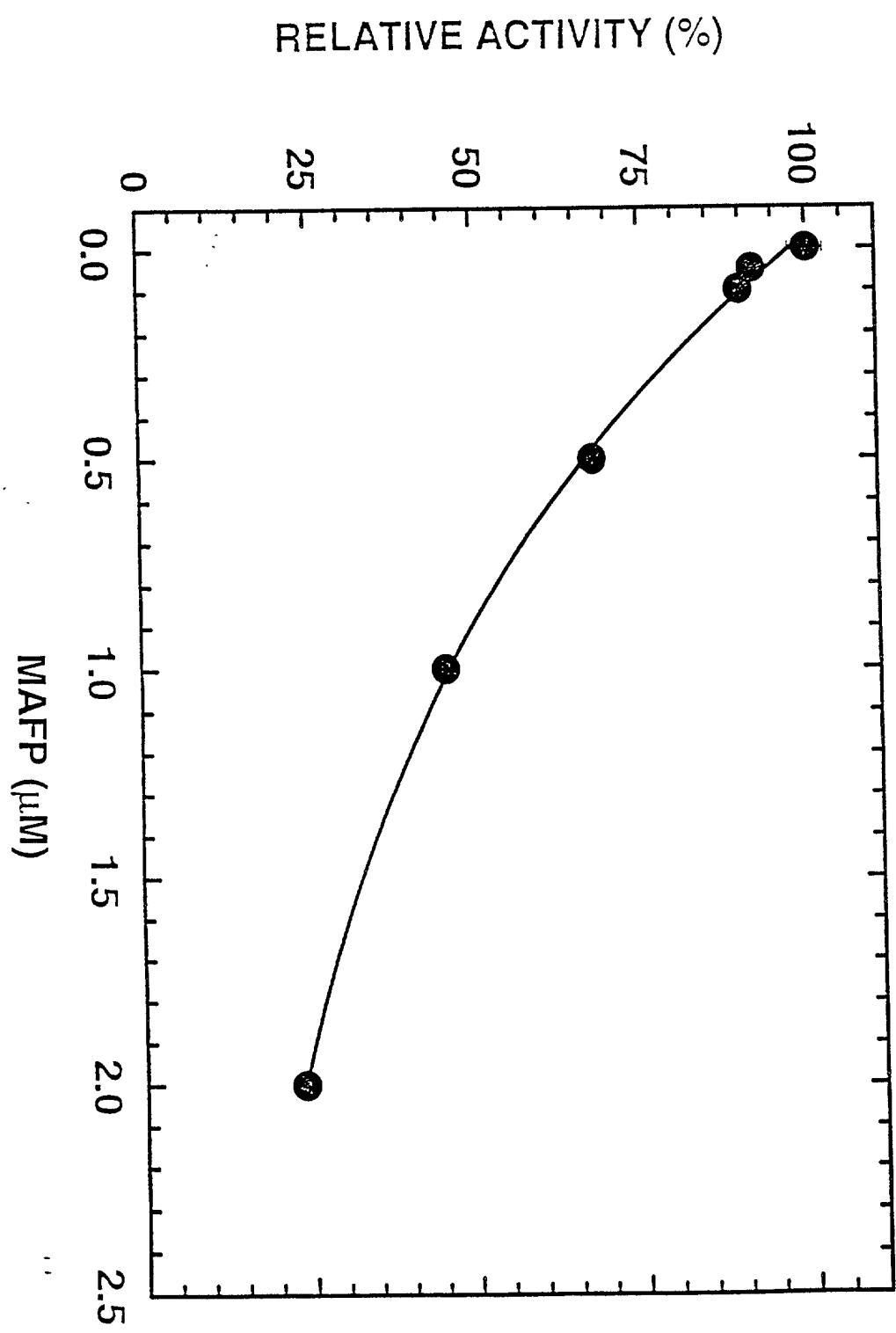


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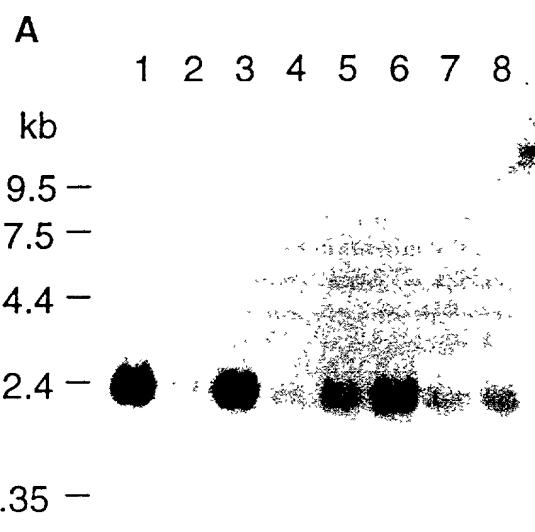


FIG 6A

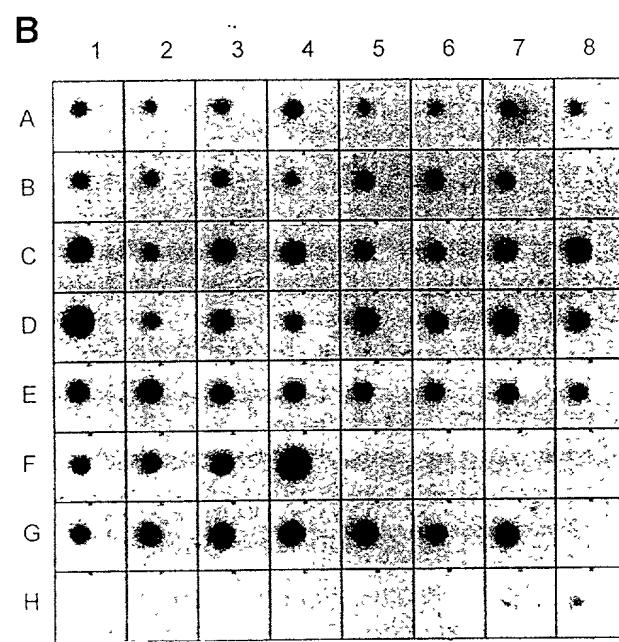


FIG 6B

C

	1	2	3	4	5	6	7	8
A	Whole Brain (8.0)	amygdala (5.0)	caudate nucleus (5.8)	cerebellum (18.0)	cerebral cortex (4.4)	frontal lobe (4.0)	hippocampus (9.6)	medulla oblongata (5.0)
B	occipital lobe (10.2)	putamen (9.2)	substantia nigra (10.9)	temporal lobe (6.7)	thalamus (17.0)	subthalamic nucleus (14.4)	spinal cord (11.2)	
C	heart (42.4)	aorta (10.7)	skeletal muscle (41.9)	colon (36.3)	bladder (17.9)	uterus (14.6)	prostate (30.8)	stomach (43.4)
D	testis (83.3)	ovary (10.8)	pancreas (31.9)	pituitary gland (16.3)	adrenal gland (46.5)	thyroid gland (25.6)	salivary gland (48.8)	mammary gland (22.8)
E	kidney (29.4)	liver (42.8)	small intestine (29.8)	spleen (31.0)	thymus (23.0)	peripheral leukocyte (21.2)	lymph node (23.0)	bone marrow (12.7)
F	appendix (15.0)	lung (21.8)	trachea (34.9)	placenta (100.0)				
G	fetal brain (18.4)	fetal heart (38.4)	fetal kidney (51.5)	fetal liver (47.7)	fetal spleen (55.5)	fetal thymus (26.9)	fetal lung (42.4)	
H	yeast total RNA 100ng	yeast tRNA 100ng	E. coli rRNA 100ng	E. coli DNA 100ng	Poly(rA) 100ng	human Cot1 DNA 100ng	human DNA 100 ng (3.4)	human DNA 500 ng (8.8)

Fig 6C

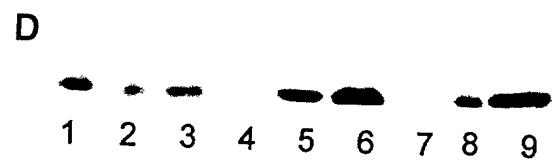


FIG 6D

[Redacted]

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
PATENT APPLICATION DECLARATION AND POWER OF ATTORNEY
(Page 1 of 2)

EACH OF THE APPLICANTS SIGNING BELOW HEREBY DECLARES THAT:

My residence, post office address, and citizenship are as stated next to my name in PART A on page 2 hereof.

I believe that I am an original, first, and joint inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled **CLONED HUMAN LYSOPHOSPHOLIPASE**, the specification of which is attached hereto.

I hereby state that I have reviewed and understood the contents of the above-identified specification, including the claims.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, Section 1.56(a).

I hereby declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent(s) issued thereon.

See next page 2 attached, signed, and made a part hereof.

PATENT APPLICATION DECLARATION AND POWER OF ATTORNEY
(Page 2 of 2)

I hereby appoint as my attorney with full power of substitution to prosecute this application and transact all business in the United States Patent and Trademark Office connected therewith:

William C. Fuess Registration Number 30,054

whose mailing address for this application is:

FUESS & DAVIDENAS
Attorneys at Law
10951 Sorrento Valley Road, Suite II-G
San Diego, California 92121-1613
Attention: William C. Fuess

and whose telecommunications numbers are:

Telephone: (619) 452-8293
Facsimile: (619) 452-8293

All correspondence and telephone calls should be directed to William C. Fuess at the address and telephone number referenced above.

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La Jolla, CA 92037

Inventor's Signature: Edward A. Dennis
Date: 1/28/00

Full Name of First Inventor: AIJUN WANG
Citizenship: U.S.A.

Residence Address: _____

Inventor's Signature: _____

Date: _____

See page 1 to which this page is attached and of which this page is a part.

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Friedman, Peter

Johnson, Christina A.

Dennis, Edward A.

<302> A Specific Human Lysophospholipase: cDNA Cloning,
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